



## Recovery effects of pomegranate seed powder on the testes following cadmium poisoning in Japanese quail (*Coturnix japonica*); a stereological and lipid peroxidation study

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### ABSTRACT

This study aimed to investigate the effects of pomegranate seed powder on cadmium-poisoned testicular tissue in Japanese quail. A total of 270 day-old Japanese quail chicks were assigned to six treatment groups, control group, cadmium 50 ppm (group II), pomegranate seed powder (1 and 0.5 %; groups III and IV, respectively), pomegranate seed powder 1% + Cd 50 ppm (group V), pomegranate seed powder 0.5% + Cd 50 ppm (group VI). Stereological parameters in testes and TBARS, cholesterol, and triglyceride levels were determined. Testicular components showed a significant reduction in area surface and volume density in the cadmium-exposed groups compared with controls ( $p < 0.05$ ). It was found that in the cadmium induction group, TBARS, cholesterol, and triglyceride levels were significantly higher compared to the normal level ( $p < 0.05$ ). The results showed that pomegranate seed powder could increase the area surface and volume density of testicular germinal ingredients and decrease the content of TBARS, cholesterol, and triglyceride ( $p > 0.05$ ) in cadmium poisoning testis. These results show that cadmium has destructive effects and pomegranate seed powder has prevented the development of these effects on stereological and lipid parameters.

### Keywords

Japanese quail, Pomegranate seed powder, Stereology, TBARS, Histology

### Abbreviations

TBARS: Thiobarbituric acid reactive substances  
Cd: Cadmium  
H&E: Hematoxylin and eosin  
ANOVA: Analysis of variance  
Spem: Spermatogonia

Spem-1: Primary spermatocyte  
Spet: Spermatid

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Cadmium is a natural heavy metal that in its industrial form, depending on its dosage, causes poisoning in humans and animals [1]. It has been reported that cadmium inflicts damage on the reproductive system of birds that can occur either experimentally [2] or by being environmentally exposed to this heavy metal [3]. Given that 1 to 2% of the acute dose of cadmium is taken up by the testes [4], however, the damage to the process of spermatogenesis is very severe [5]. Low doses of cadmium alter the immunological microenvironment in the testicles, which results in increased testicular autoimmunity [6]. Testicular poisoning with cadmium is caused by complex intra-network reactions, including the destruction of the blood testicular barrier or inhibition of oxidative stress leading to germ cell apoptosis, edema, and intra-testicular bleeding [7-9].

Pomegranate (*Punica granatum*) fruit, juice, seed, and peel due to a high content of polyphenols, especially ellagitannins, tannins, and anthocyanins have high antioxidant capacities [10]. Consumption of pomegranate extract significantly increases the sperm quality, density of the spermatogenic cell, antioxidant activity, and testosterone levels in male rats [11]. It has also been shown that pomegranate contains hydrolyzed and concentrated tannins that exhibit anti-cancer properties in vitro and in vivo [12, 13].

Qualitative patterns, including atrophy, hypoplasia, hyperplasia, and or hypertrophy can be stated by stereological methods through measurable and comparative data [14]. However, the impact of various situations on the process of spermatogenesis and or survival of the germ cell line requires simple methods and quantification. This study aimed to determine the protective effects of pomegranate seed powder against the toxic effects of Cd in quail using biochemical and histo-stereological methods. All the chemicals and reagents used in this study were of molecular biology grade and were purchased from Sigma-Aldrich Co. Ripe pomegranates are purchased from the local market and after separating the skin, seeds (protein, 13.6; fat, 29.6, fiber, 39.3) were washed with water and after drying, seeds were powdered using a hammer mill and sieved through a 40-mesh sieve [15]. All protocols used in this experiment were approved by the Institutional Animal Care and Use Committee of Shahrekord University. Two hundred and seventy-one-day-old unsexed quail (Japanese) chicks were used in this study. The birds were fed by the standard basic diet until the 7th day of age (one week, habituation) before starting the experiment. All diets were formulated to meet the nutrient requirements of the Japanese quails. On day 7, the birds were randomly assigned to six treatments, with three replicates (cages) of fifteen quails each.

Group I: received basic diet and ordinary water. Group II: received a basic diet and 50 ppm cadmium orally. Group III: birds were fed with a basic diet + 1% pomegranate seed powder. Group IV: birds were fed with a basic diet + 0.5% pomegranate seed powder. Group V: received a basic diet along with 1% pomegranate seed powder and 50 ppm cadmium. Group VI: received basic diet along with 0.5% pomegranate seed powder and 50 ppm cadmium.

Blood samples were centrifuged at 1500 rpm for 15 minutes and TBARS, cholesterol, and triglyceride in serum samples were measured. Testes specimens fixed in Merck formalin and processed through paraffin embedding, cut into 5 µm sections, stained with hematoxylin and eosin (H&E) techniques for stereological evaluation.

Stereology

By the count of all point, the total area determined (Figure 1), and finally the volume was estimated by:

$$V = k \times t \times a(p) \times \sum^p(\text{testis})$$

In which  $\sum^p$  is total area, k is  $k^{\text{th}}$  section is mounted on a glass slide,  $a(p)$  is the area of each point.

The volume of the seminiferous tubules, the inter-tubular tissues, and the germinal epithelium was estimated [16] by:

$$V_{V(\text{structure}/\text{ref})} = P_{(\text{structure})} / P_{(\text{total})}$$

In which  $P_{(\text{structure})}$  and  $P_{(\text{total})}$  represent respectively, the total numbers of points hitting the structures of interest, and the reference space  $V_{(\text{ref})}$  and summed over all sections and fields from one testis.

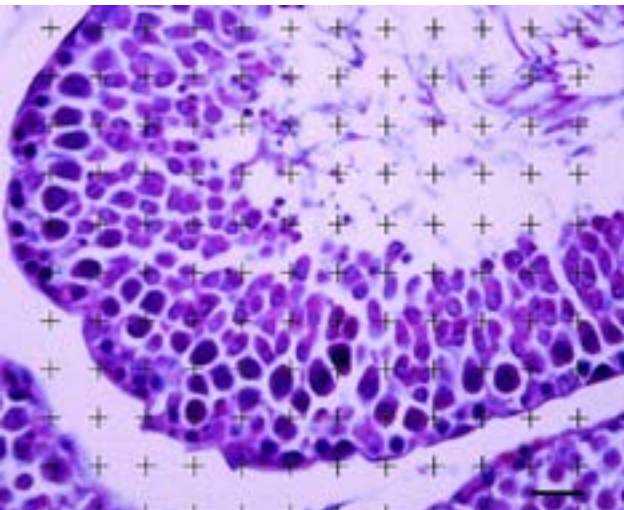


Figure 1. Photomicrograph of testis in quail. Point-counting method was used to evaluate stereological parameters. The area surface and volume density of the spermatogonia, primary spermatocyte, and spermatid were determined by calculation of the points hitting with the micrograph; (scale bar=20 µm).

$$V_{(\text{structure})} = V_{V(\text{structure}/\text{testis})} \times V_{\text{final}}(\text{mm}^3)$$

The total volume was obtained by multiplying the density by the final testis volume [16].

Biochemical analysis

TBARS was determined calorimetrically using the method of Buege and Aust (1978), briefly, 0.1 mL of serum was treated with 2 mL of TBA-TCA-HCl reagent, and absorbance of the supernatant was measured against reference blank at 535 nm. Concentrations were calculated using an extinction coefficient of  $1.56 \times 10^5 \text{ mol}^{-1} \text{ L cm}^{-1}$  and expressed as  $\text{nmol mL}^{-1}$  [17]. Blood biochemical parameters including triglyceride and cholesterol were measured by commercial kits of Pars Azmoun Company and according to the protocols of this company. Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS V. 23/0 software (SPSS Inc., Chicago, IL). The means were considered significantly different at  $p < 0.05$ .

The Vv (spem/tes) (Table 1) was significantly decreased in the cadmium-exposed group compared to the control group ( $p < 0.05$ ). In addition, the Vv (spem-1/tes) showed a significant decrease in groups that received cadmium compare to the control group ( $p < 0.05$ ). The Vv (spet/tes) also were significantly decreased in the group exposed to the cadmium compared to the control groups ( $p < 0.05$ ). Nevertheless, the administration of pomegranate seed powder (1 and 0.5%) along with cadmium (50ppm, V and VI groups) inverted the mean of all stereological volumetric parameters to near normal. But these differences between V and VI groups with the control group were not statistically significant ( $p > 0.05$ ).

The area surface (Table 1) in all stereological parameters of spermatogonia, primary spermatocytes, and spermatids was decreased in the cadmium groups

compared to the control birds ( $p < 0.05$ ). Following a similar pattern of volume density, pomegranate seed powder (1 and 0.5%) reversed the area surface of the measured parameters mentioned non-significantly toward normal in cadmium-exposed quails (V and VI groups;  $p > 0.05$ ).

The mean TBARS level (Figure 2 and Table 2) of the control group (group I) compared with those of the other groups had no significant difference in this study except for the cadmium group (group II). Nevertheless, a significant increase had happened in the cadmium group ( $32.27 \pm 4.32$ ) in comparison with the TBARS level of the control group ( $20.3 \pm 3.21$ ;  $p < 0.05$ ). On the other hand, pomegranate seed powder (1 and 0.5%) was able to bring the level of TBARS in cadmium poisoned quail closer to the control group, although this reduction was not significant ( $p > 0.05$ ).

It should be noted that there was a significant increase in the cholesterol and triglyceride values (Table 2) of the cadmium group ( $181.41 \pm 7.28$  and  $277.08 \pm 86.16$  respectively) compared with those of the control group ( $148.99 \pm 25.07$  and  $194.78 \pm 7.49$  respectively;  $p < 0.05$ ). Pomegranate seed powder (1 and 0.5%) groups (groups V and VI) showed a decrease in cholesterol and triglyceride values in cadmium-poisoned birds' groups, although this decrease was non-significant ( $p > 0.05$ ). Therefore, the administration of pomegranate seed powder in two different doses ( $p > 0.05$ ) reversed the levels of cholesterol and triglyceride to near normal in cadmium-exposed birds.

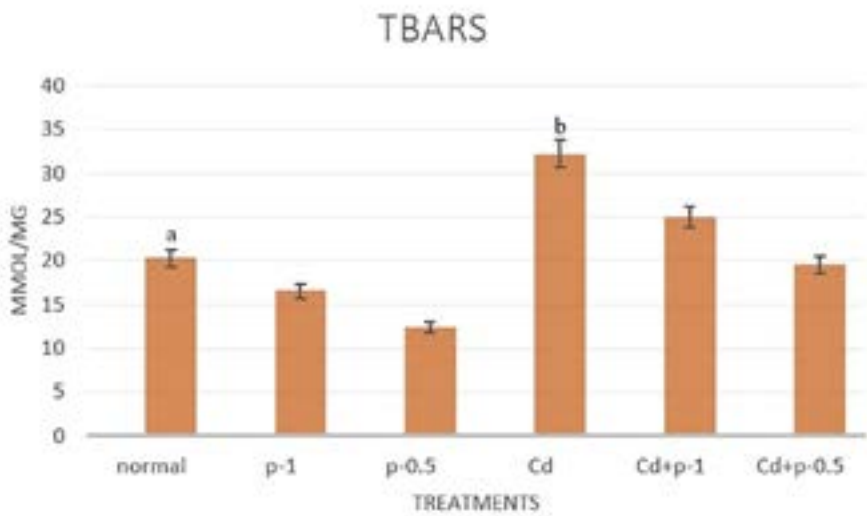
This study showed that the volume density of spermatogonia, primary spermatocyte, and spermatid affected by cadmium administration in a dose of 50 ppm. Meanwhile, similar changes were observed regarding the area surface of structural components mentioned in the face of cadmium. As the results showed, a significant decrease was seen in area sur-

Table 1. Effect of pomegranate on stereological parameters in the testes of cadmium-exposed birds

		Area surface			Volume density		
		spem	spem-1	spet	spem	spem-1	spet
I	Control	0.16 ± .07 <sup>a</sup>	0.21 ± 0.10 <sup>a</sup>	0.17 ± .04 <sup>a</sup>	0.25±0.13 a	0.43 ±.17 <sup>a</sup>	0.25 ± 0.13 <sup>a</sup>
II	Cd	0.07 ± .04 <sup>b</sup>	0.12 ± .05 <sup>b</sup>	0.10 ±.06 <sup>b</sup>	0.10±.04 b	0.23 ±.19 <sup>b</sup>	0.10 ±.04 <sup>b</sup>
III	Pome. 1%	0.13 ± .03	0.23 ± .06	0.17 ±.06	0.28±.13	0.50 ±.26	0.28 ±.13
IV	Pome. 0.5%	0.13 ± .06	0.23 ± .07	0.20 ±.07	0.24±.19	0.50 ±.25	0.24 ±.19
V	Pome. 1% + Cd	0.12 ± .03	0.21 ± .08	0.14 ±.04	0.22±.13	0.41 ±.17	0.22 ±.13
VI	Pome. 0.5% + Cd	0.12 ±.02	0.20 ± .07	0.14 ±.07	0.21±.12	0.41 ±.18	0.21 ±.12

<sup>a, b</sup> Significant difference compared with controls in each column at  $p < 0.05$ ; Spem: spermatogonia, Spem-1: primary spermatocytes, Spet: spermatid.





**Figure 2.** Effect of pomegranate on TBARS levels of testes of cadmium-exposed birds. Groups: normal (control), p-1 (pomegranate 1%), p-0.5 (pomegranate 0.5%), Cd (cadmium), Cd+p-1 (cadmium+ pomegranate 1%), Cd+p-0.5 (cadmium+ pomegranate 0.5%); Dissimilar letters indicate significant differences.

face and volume density in the cadmium-treated birds in comparison with those of the control group. In agreement with our findings, Blanco et al. displayed a significant decreasing in the volume density of the testicular structure and decreasing tubular densities and epithelial percentages in the animals that were exposed to cadmium [18]. Adamkovicova et al. showed that the decrease in the epithelial layer volume of the seminiferous tubules in the face of cadmium can be due to the penetration of cells into the lumen leading to lumen shrinkage and a significant decrease in the percentage of epithelial volume fraction [19]. Therefore, we infer that a decrease in the volume density of measured parameters in our study could be another reason for the reduction of the epithelium layer volume in Adamkovicova's research. Numerous studies have shown that alteration in quantitative parameters, similar to measured parameters of this study, is related to toxic effects of cadmium which can result in the miss of germination epithelium [20, 21].

The results of this study show that no significant

differences were observed between the control group and the pomegranate seed powder group in all the evaluated parameters. The results of this study also revealed that the area surface and the volume density of spermatogonia, primary spermatocyte, and spermatid were increased in groups V and VI compared to the cadmium group.

Cadmium exposure for 35 days caused higher TBARS, cholesterol, and triglyceride values in the serum of exposed birds to cadmium in the comparison control group. These are according to other findings that cadmium produces exceeding amounts of reactive oxygen species (ROS), which leads to oxidative stress damage and causes of injury and disease expansion [9, 22-26]. An increment in TBARS capacity was considered as a sign of oxidative damage [27, 28]. The present study showed that TBARS value in group II increases than control, but in groups V and VI, the TBARS value was at a low level compared with group II. Therefore, oral administration of pomegranate seed powder (both 1 and 0.5%) to cadmium treated

quails non-significantly ( $p > 0.05$ ) decreased the altitude of TBARS when compared to the group treated with cadmium alone. Administration of pomegranate seed powder reversed the concentration of TBARS in cadmium exposed birds; such that, this shift caused the amount of TBARS value to deviate to the normal level, although these changes were not significant. Like TBARS, similar results were obtained in the cholesterol and triglyceride levels. It was observed that the levels of cholesterol and

**Table 2.** Effect of pomegranate on biochemical parameters in the serum of cadmium-exposed birds

Groups		parameters		
		TBARS mmol/mg	Cholesterol mg/d	Triglyceride mg/d
I	Control	20.30 ± 3.21 <sup>a</sup>	148.99 ± 25.07 <sup>a</sup>	194.78 ± 7.49 <sup>a</sup>
II	Cd	32.27 ± 4.32 <sup>b</sup>	181.41 ± 7.28 <sup>b</sup>	277.08 ± 86.16 <sup>b</sup>
III	Pome. 1%	16.57 ± 2.17	139.36 ± 17.45	152.37 ± 21.16
IV	Pome. 0.5%	12.35 ± 2.01	123.32 ± 15.37	142.10 ± 15.42
V	Pome. 1% + Cd	25.00 ± 2.64	153.78 ± 24.26	224.34 ± 24.61
VI	Pome. 0.5% + Cd	19.58 ± 2.85	168.95 ± 16.56	208.27 ± 28.14

<sup>a,b</sup> Significant difference compared with controls in each column; Each value represents the mean ± SEM

triglyceride in the serum exposed to cadmium had an incremental distribution pattern than to controls; vice versa, the amount of these parameters in groups V and VI showed a declining process when compared with the cadmium-induced group (II).

Saleh et al. (2018) have reported that the phenolic compounds present in the pomegranate peel powder and extract may have the same antioxidant activity as  $\alpha$ -tocopherol acetate in the meat of broiler chickens and decreased the oxidation rate and TBARS index, although their mechanism of action may be different [29]. The results of the present study indicated that the TBARS content in samples treated with the pomegranate powder was non significantly lower than that of the Cd group. In conclusion, these results indicate that Cd induces destructive effects on the stereological indices and lipid peroxidation markers and pomegranate seed powder has prevented the development of these effects in the testis of Japanese quails.

Authors' Contributions

RG, RA and HM performed the experiments. RF, IK, and BK designed the research project and drafted the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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